Poeyana
REVISTA CUBANA DE ZOOLOGÍA
ISSN 2410-7492
RNPS 2403
Acceso Abierto
http://revistas.geotech.cu/index.php/poey
COMUNICACIÓN CORTA
510 (enero -junio 2020): 72 - 78

First record of *Bipalium nobile* Kawakatsu & Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) for Aichi Prefecture, Japan

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Abstract: The land planarian *Bipalium nobile* Kawakatsu & Makino, 1982 is recorded by first time for Aichi Prefecture, Japan. Four immature specimens were identified based on external morphology, histology and two molecular markers (28S rDNA and COI). The shape of the body and color pattern agreed with the original description of the species. Internally, the arrangement of the cutaneous and parenchymal musculature at level of the pre-pharyngeal region also agree with the description of *B. nobile*. Phylogenetic analyses based on the 28S rDNA shown that the present individuals form a monophyletic clade with other available sequences from Japan and South Korea. The COI sequences are the first obtained for *B. nobile* from Japan.

Keywords: 28S rDNA, DNA barcoding; land planarian, new locality

Resumen: Primer registro de *Bipalium Nobile* Kawakatsu *et* Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) para la prefectura de Aichi, Japón. La planaria terrestre *Bipalium nobile* Kawakatsu & Makino, 1982 se registró por primera vez para la prefectura de Aichi, Japón. Cuatro individuos juveniles fueron identificados mediante caracteres externos, histología y dos marcadores moleculares (28S rDNA y COI). La forma del cuerpo y el patrón de coloración coinciden con la descripción original de la especie. Internamente, la disposición de la musculatura cutánea y parenquimática al nivel de la región prefaríngea también coinciden con la descripción de *B. nobile*. El análisis filogenético basado en el marcador 28S rDNA mostró que los individuos del presente estudio forman un clado monofilético con otras secuencias de Japón y Corea del Sur. Las secuencias de COI fueron las primeras de este gen obtenidas para especímenes de *B. nobile* de Japón.

Palabras Clave: 28S rDNA, código de barra, nueva localidad, planaria terrestre.

Introduction

The land planarian sub-family Bipaliinae presents a high diversity in the Japanese archipelago, with about 20 species recorded, belonging to the genera *Bipalium* Stimpson, 1857; *Diversibipalium* Kawakatsu, Ogren, Froehlich & Sasaki, 2002 and *Novibipalium* Kawakatsu, Ogren & Froehlich, 1998 (Kawakatsu *et al.*, 2005). One of these species: *Bipalium nobile* Kawakatsu & Makino, 1982 was described from Tokyo area and is remarkable by its large body length, up to 1 m in adult specimens (Kawakatsu *et al.*, 1982). Subsequently, *B. nobile* has been recorded from several localities in Honshu, Kyushu and Shikoku islands (Kawakatsu *et al.*, 1998; Kawakatsu *et al.*, 2005; Murayama and Kawakatsu, 1999).

In the current study, *B. nobile* is recorded by the first time from Aichi prefecture based on external morphology, histology and 28S rDNA and COI of immature specimens. The COI sequences obtained constitute the first from Japanese specimens of *B. nobile*.

Material and Methods

Specimen collecting and fixation

Four specimens of a land planarian attributed to *Bipalium nobile* were collected by hand under litter from the Chubu University Campus,

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Publicado en línea el 30 de junio de 2020

Kasugai, Aichi prefecture, Japan and kept alive in jars with moistened litter until processing. They were measured alive with a ruler (± 1 mm) and photographed with a Nikon D5300 camera with an AF-S DX Micro NIKKOR 85 mm macro lens (Nikon, Japan). One individual was directly killed in 100% ethanol for DNA studies. The rest of the flatworms were killed with hot water and a small fragment of the posterior end was cut from one specimen and stored in 100% ethanol for molecular purposes. Then, they were fixed in 4% neutral phosphate buffered formaldehyde and transferred to 70% ethanol for storage. Voucher specimens are deposited in the Colección Helmintológica de las Colecciones Zoológicas (CZACC), Instituto de Ecología y Sistemática, Havana, Cuba.

Histological studies

Two specimens were selected for histological studies. Tissue blocks of the pre-pharyngeal region and pharynx were dehydrated in an ascending ethanol series and embedded in paraffin wax. Transversal (pre-pharyngeal region) and sagittal (pharynx) 5-7 μm sections were stained with Cason´s Mallory-Heidenhain trichrome (Winsor and Sluys, 2018) and mounted in Canada balsam or Permount. For the pre-pharyngeal region, Cutaneous Musculature Index (CMI) (Froehlich, 1955) and Parenchymal Musculature Index (PMI) (Winsor, 1983) were calculated. Micrographs were taken with an AxioCam digital camera attached to a Carl Zeiss Axioskop 2 Plus compound microscope. Scale bars of all plates are given in micrometers. For the observation of the eyes arrangement, one specimen was dehydrated in an ethanol series and isopropyl series and cleared in clove oil.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted with the DNEasy® Blood & Tissue kit (Qiagen, USA) following manufacturer´s protocol. The 28S rDNA gene was amplified in two fragments using the primers 28S_1F (5´-TAT CAG TAA GCG GAG GAA AAG-3´) and 28S_4R (5´ -CCA GCT ATC CTG AGG G-3´) with the overlapping primer set 28S_2F (5´-CTG AGT CCG ATA GCA AAC AAG-3´) and 28S_6R (5´-GGA ACC CCT TCT CCA CTT CAG T-3´) (Álvarez-Presas *et al.*, 2008). Partial sequences (~900 bp) of the cytochrome c oxidase subunit I, COI gene were amplified with the primer set BarS [5´-GTT ATG CCT GTA ATG ATT G-3´ (Álvarez-Presas *et al.*, 2011)] and COIR [5´-CCW GTY ARM CCH CCW AYA GTA AA-3´ (Lázaro *et al.*, 2009)].

PCR reactions were performed with the KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). For the 28S rDNA the total volume of the reactions was 30 μL, with the PCR cycling parameters consisting of an initial denaturation at 94ºC by 1 min followed by 35 cycles of 98ºC by 10 s, 50ºC by 30 s and 68ºC by 1 min and a final extension step of 68ºC by 5 min. For the COI gene the total volume was 20 μL with the PCR cycling parameters consisting of an initial denaturation at 94ºC by 2 min followed by 35 cycles of 98ºC by 10 s, 45ºC by 30 s and 68ºC by 30 s and a final extension step of 68ºC by 5 min. The results of the PCR reactions were checked by agarose gel electrophoresis. Then PCR products were excised from gel and purified with the NucleoSpin Gel and PCR Clean Up kit (Macherey-Nagel, Düren, Germany), following manufacturer´s protocol. Samples were submitted to Hokkaido System Science Co., Sapporo, Japan. The original PCR primers were used to sequence both strands. Raw sequences were manually edited with Sequencher 4.1.4 (http://genecodes.com) and deposited in GenBank NCBI (http://www.ncbi.nlm.nih.gov/genbank/), with the accession numbers designated in the phylogenetic tree.

Phylogenetic analyses

Several 28S rDNA sequences of bipaliin species were selected from GenBank for the phylogenetic analyses (accession numbers in the phylograms). Four species of the genus *Microplana* (Microplaninae) were used as outgroup. Multiple sequence alignments were made using the Muscle algorithm with default parameters as implemented in MEGA6 (Tamura *et al.*, 2013). The poorly aligned regions and gaps were automatically removed with trimAl (Capella-Gutiérrez *et al.*, 2009). MEGA6 was used to estimate the optimal model of sequence evolution (HKY+G) following the Bayesian Information Criterion (BIC). The Maximum likelihood analysis (ML) was also performed with MEGA6. Branch support for the ML tree was inferred by bootstrap resampling using 1,000 iterations. Bayesian Inference analysis (BI) was performed with MrBayes v3.2.6 (Ronquist *et al.*, 2012), with 5×10^6 generations, sampling every 100 generations and discarding the first 25% of the sample runs as burn-in. The convergence statistics of the BI process sta-

tionarity and the number of burn-in trees were checked using Tracer v1.5 (Rambaut *et al.*, 2003).

Many of the COI sequences of bipaliins from GenBank (including the one available of *B. nobile*) are very short. Due to that, most of the information is missing after the alignment and trimming of the dataset and the support values of the nodes are low, as was observed in previous studies (Justine *et al.*, 2019). That was the reason why the phylogenetic analyses were based only on the larger 28S rDNA.

Results and Discussion

Material examined

Voucher CZACC 18.003; Japan, Aichi prefecture, Kasugai, Chubu University Campus; 35º16´28.315´´N, 137º1´3.259´´E; V/2016; J. Morffe coll.; entire specimen killed directly in 100% ethanol, a small portion of the posterior end removed for molecular studies.

Voucher CZACC 18.004; Japan, Aichi prefecture, Kasugai, Chubu University Campus; 35º16´28.315´´N, 137º1´3.259´´E; V/2016; J. Morffe coll.; pre-pharyngeal region, transverse sections on 3 slides; pharynx, sagittal sections on 4 slides; rest of the specimen stored in 70% ethanol; a small portion of the posterior end removed for molecular studies.

Voucher CZACC 18.005; Japan, Aichi prefecture, Kasugai, Chubu University Campus; 35º16´28.315´´N, 137º1´3.259´´E; V/2016; J. Morffe coll.; pre-pharyngeal region, transversal sections on 3 slides; pharynx, sagittal sections on 3 slides; rest of the specimen stored in 70% ethanol.

Voucher CZACC 18.006; Japan, Aichi prefecture, Kasugai, Chubu University Campus; 35º16´28.315´´N, 137º1´3.259´´E; V/2016; J. Morffe coll.; entire specimen stored in 70% ethanol.

Morphological examination

The four specimens collected were immature, measuring in length 50-110 mm alive, with the mouth distant from the anterior end 27.9%-39.0% of the body length; a gonopore was absent. The head is semilunate in living specimens, contracted and rounded in heat-fixed individuals. The ground color of the dorsum is a uniform light yellowish brown. The head exhibits a darker brownish color with paler margin. Five dorsal stripes are present: a median, dark stripe with well-defined margins that extends from the anterior third of the head to the posterior end of the body; two paired lateral stripes, with diffuse margins, slightly wider and paler than the median one, extend from the base of the head to the posterior end. Finally, two marginal stripes extend from the base of the head to a just before the level of the mouth. The ventral region of the body is yellowish, with a whitish creeping sole, flanked by two purplish stripes (Fig. 1).

In the head plate, the eyes form a dorsal band around the entire margin, presenting several rows of minute eyes, clustered at the anterior margin of the head and becoming more scattered towards the posterio-lateral margin (Fig 2). On both sides of the neck, the eyes are ventrally displaced, forming a band of four to five rows of eyes that become narrow at the posterior half of the body. The eyes are absent at the posterior end.

Internally, the cutaneous musculature is very reduced (CMI = 1.79% - 1.90%), consisting of two layers, an outer circular layer and an inner longitudinal layer of muscles (Fig. 3A, B). Beneath the nerve plexus adjacent to the longitudinal cuticular muscle fibres there is a ring-zone consisting of intermingled longitudinal and circular fibres, PMI = 12.8%-13.3%. The pharynx is plicate collar-form (Fig. 3C).

The external morphology of our specimens agrees with the original description of *B. nobile* (Kawakatsu *et al.*, 1982). According to Kawakatsu *et al.* (1982), the sexually mature specimens of *B. nobile* range in size from 320 mm to *ca.* 1,000 mm in total body length. The values for the position of the mouth relative to the total body length in our specimens are more posterior than the ones obtained by Kawakatsu *et al.* (1982) for large, sexually mature individuals: one-fifth to one-sixth of the body length. However, the same authors recorded that in middle-sized specimens the mouth is located more posterior than in large specimens, coinciding with our results. The eye pattern is consistent with *B. nobile* (Fig. 2). Unfortunately, Kawakatsu *et al.* (1982) did not calculate the CMI or PMI for body musculature, and so these cannot be calculated from data they provide. However, the structure and relative thicknesses of the cutaneous and parenchymal muscles in pre-pharyngeal transverse sections of our specimens agrees closely with the description, figure and photo in Kawakatsu *et al.* (1982) of the same

Figure 1. *Bipalium nobile* Kawakatsu & Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) from the Chubu University Campus, Aichi prefecture, Japan (specimen CZACC 18.006): A, habitus; B, head. Scale lines not available. Photos © J. Morffe.

Figura 1. *Bipalium nobile* Kawakatsu *et* Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) del Campus de la Universidad de Chubu, prefectura de Aichi, Japón (ejemplar CZACC 18.006): A, ejemplar completo; B, cabeza. Líneas de escala no disponibles. Fotografías © J. Morffe.

region in *B. nobile*, and with the *Diversibipalium virgatum* group of Winsor (1983) to which *B. nobile* belongs, characterized by weak cutaneous musculature (CMI = 1%) comprising only circular and longitudinal muscle layers, and strong parenchymal musculature (PMI = 8% - our specimens have PMI = 12.9%-13.3%). Both *B. nobile* and our specimens share the same collar-form pharyngeal type.

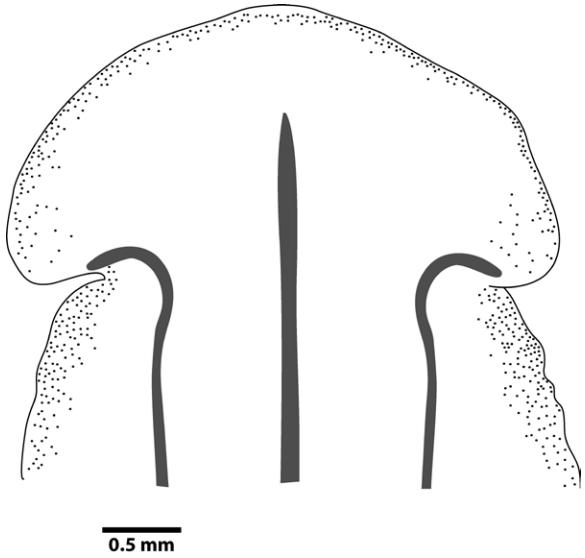

Figure 2. *Bipalium nobile* Kawakatsu & Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) from the Chubu University Campus, Aichi prefecture, Japan. Dorsal eye pattern at level of the head plate.

Figura 2. *Bipalium nobile* Kawakatsu *et* Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) del Campus de la Universidad de Chubu, prefectura de Aichi, Japón. Disposición de los ojos en la superficie dorsal de la cabeza.

DNA studies

Two identical partial sequences of the 28S rDNA gene (1556 bp and 1573 bp, respectively) were obtained. The ML and BI trees showed identical topology, thus only the ML tree is shown (Fig. 4). The phylogenetic analyses show that the present specimens form a monophyletic clade with other available sequence of *B. nobile* from Japan (DQ665958) and with a South Korean specimen (HM346596) identified as *B. nobile* by Moon *et al.* (2011). This points that the studied population is conspecific with the Japanese DQ665958 specimen. Moreover, two identical partial sequences of the COI gene were obtained from the studied individuals: MG436935 (757 bp) and MG436936 (762 bp). Such sequences differ in seven bp (in an alignment of 324 bp) with a South Korean specimen (HM346596). According to the data available at GenBank, such COI sequence appear to belong to the same specimen from which the 28S rDNA marker was obtained.

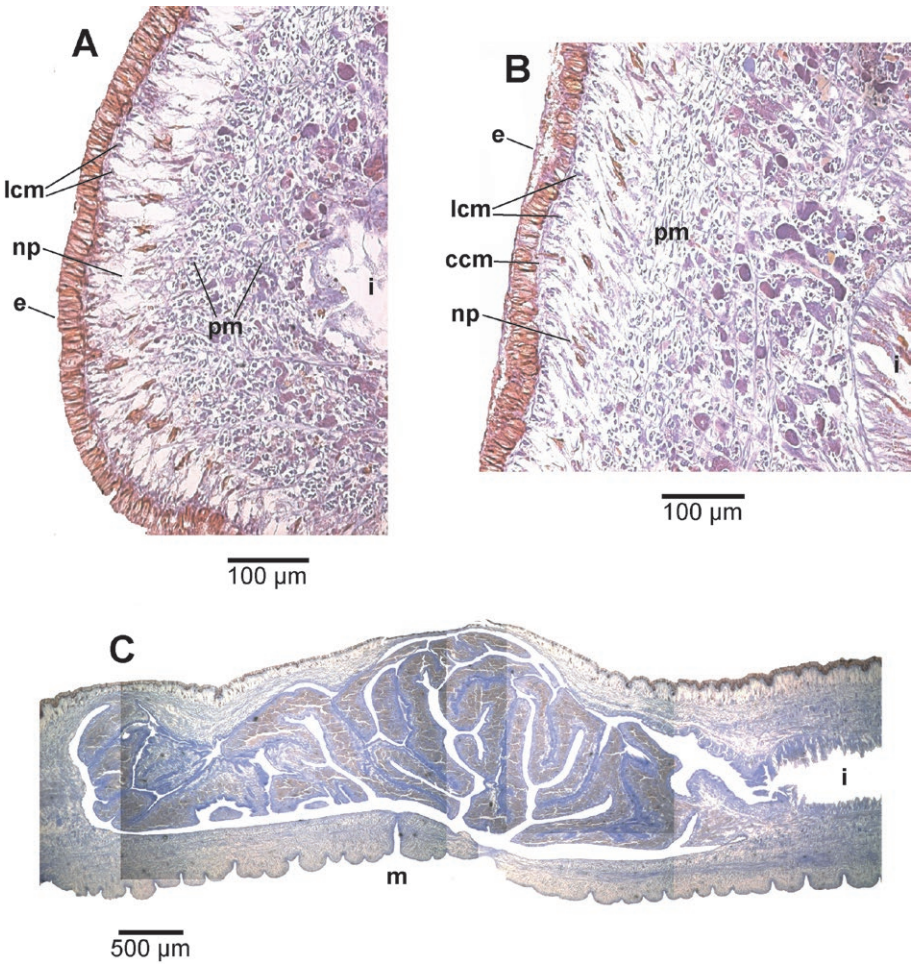

FIGURE 3. *Bipalium nobile* Kawakatsu & Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) from the Chubu University Campus, Aichi prefecture, Japan: A, B, transverse sections of the pre-pharyngeal region; C, sagittal section of the pharynx. Abbreviations: ccm = cutaneous circular muscles; e = epidermis; i = intestine; lcm = longitudinal cutaneous muscles; m = mouth; np = nerve plexus; pm = parenchymal musculature.

FIGURE 3. *Bipalium nobile* Kawakatsu & Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) del campus de la Universidad de Chubu, Prefectura de Aichi, Japón: A, B, sección transversal al nivel de la región prefaríngea; C, sección longitudinal de la faringe. Abreviaturas: ccm = musculatura circular cutánea; e = epidermis; i = intestino; lcm = musculatura longitudinal cutánea; m = boca; np = plexo nervioso; pm = musculatura parenquimática.

REMARKS

According to Kawakatsu *et al.* (2001) the natural range of *B. nobile* is central and southern China and was introduced in Japan after World War II. Considering such natural range, the species also could be introduced in Korea from southern China. The pictures of the living specimens identified as *B. nobile* by Moon *et al.* (2011) show similar features to the Japanese individuals, namely a semilunate head, the dorsal ground color of a light yellowish brown, the head of a darker brownish color and three visible stripes: a median, dark, well-defined stripe that starts from the anterior third of the head and two paired lateral stripes, with diffuse margins, starting from the base of the head. Otherwise, there are not data on other external features (e.g. total length, position of the mouth and presence and position of the gonopore) or histology that could help to a more accurate identification. The p-distance for the COI gene between the Japanese and South Korean sequences is 0.7%, for the alignment of 324 bp. However, due to the similarities that exist among several species of Bipaliinae and the difficulties for the identification to specific level without the histological study of sexually mature specimens we cannot ascertain that the South Korean specimen belong to *B. nobile*. New collects of sexual specimens from the area and further integrated anatomical and molecular studies are needed in order to assure that these are,

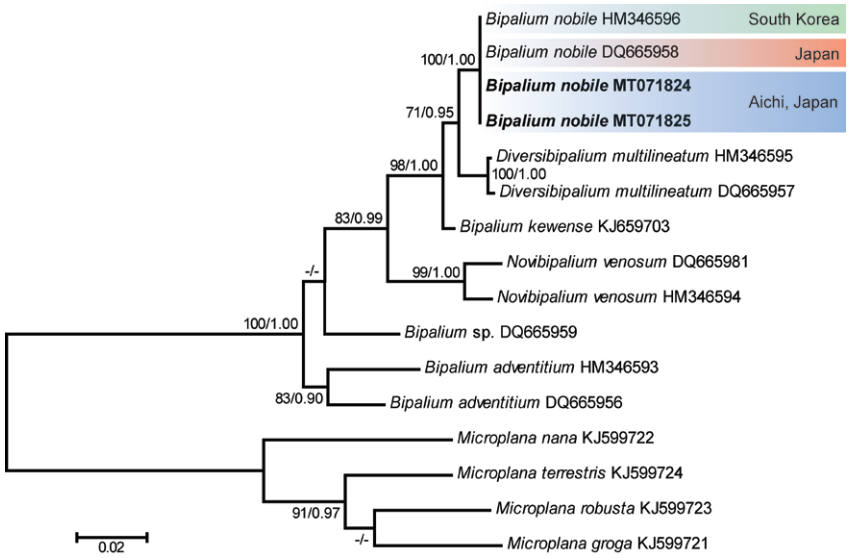

Figure 4. Maximum likelihood phylogram inferred from 28S rDNA gene for several species of the subfamily Bipaliinae (Tricladida: Geoplanidae). Four *Microplana* species were used as outgroup. Values at the nodes correspond to bootstrap resampling (≥70)/posterior probability (≥0.90). Newly obtained sequences in bold.

Figura 4. Filograma de máxima verosimilitud para el marcador molecular 28S rDNA para varias especies de la subfamilia Bipaliinae (Tricladida: Geoplanidae). Cuatro especies de *Microplana* se emplearon como grupo externo. Los valores en los nodos corresponden al bootstrap (≥70)/probabilidad posterior (≥0.90). Las nuevas secuencias obtenidas se muestran en negrita.

in fact, conspecific with the Japanese *B. nobile*. The COI sequences of the current study constitutes the first of this gene obtained for *B. nobile* from Japan.

B. nobile has been recorded from several localities across the Japanese archipelago (Fig. 5), e.g. Nagasaki prefecture in Kyushu Island (the southernmost record), Kochi and Kagawa prefectures in Shikoku Island. On Honshu Island, the species is present in the prefectures of Akita, Chiba, Gunma, Kagawa, Kanagawa, Niigata, Osaka, Tokyo, Toyama and Yamaguchi (Kawakatsu *et al.*, 1998; Kawakatsu *et al.*, 2005; Murayama and Kawakatsu, 1999). Akita prefecture constitutes the northernmost record. The Chubu University Campus, in the city of Kasugai, Aichi prefecture constitutes a new locality record for the species, separated by *ca.* 150 km from Toyonaka, Osaka prefecture (Kawakatsu *et al.*, 1998), the closest record.

The present specimens were found under a thick layer of litter in a garden of the Chubu University Campus, in a humid and shaded area. This agrees with other authors (*i.e.* Kawakatsu *et al.*, 1982; Murayama and Kawakatsu, 1999) who collected the species in gardens and other town areas. This supports the statements of Kawakatsu *et al.*, (2001) about the dispersion of *B. nobile* due to the trade and distribution of potted garden plants.

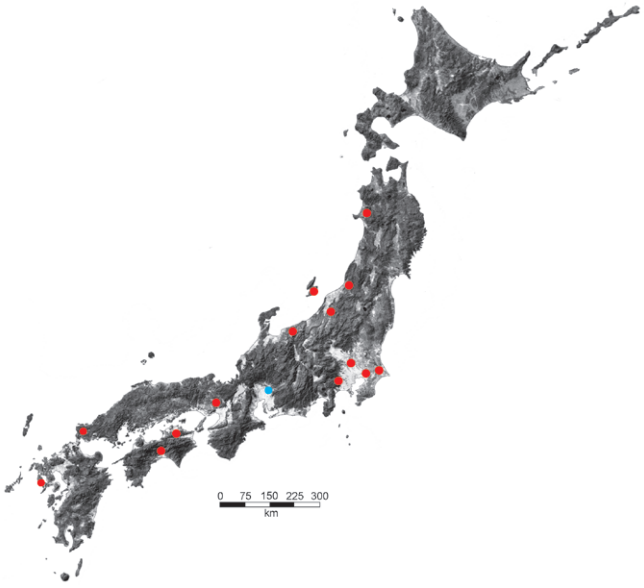

Figure 5. Records of *Bipalium nobile* Kawakatsu & Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) in Japan. The blue circle corresponds to the present record for Chubu University Campus, Aichi prefecture and the red circles correspond to previous records.

Figura 5. Registros de *Bipalium nobile* Kawakatsu *et* Makino, 1982 (Tricladida: Geoplanidae: Bipaliinae) en Japón. El círculo azul corresponde al presente estudio para el Campus de la Universidad de Chubu, prefectura de Aichi. Los círculos rojos corresponden a registros previos.

ACKNOWLEDGEMENTS. We thank to the staff of the laboratories of Pathological Anatomy of the Institute of Gastroenterology and the University Hospital "General Calixto García", Havana, Cuba for their help with the histological processing. We are grateful to Leigh Winsor (College of Science and Engineering, James Cook University) for his critical review of the manuscript and useful comments. Byron Adams (Brigham Young University and Monte L. Bean Museum) provided comments on early drafts of the manuscript. This work was supported by the Japanese Society for the Promotion of Science (JSPS) Long Term Fellowship [ID No. L16566], awarded to JM and the Research Institute for Biological Function, Chubu University (KH).

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Recibido: 22 de mayo de 2020/aceptado: 16 de junio 2020